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Single-Colony Derived Strains of Human Marrow Stromal Fibroblasts Form Bone After Transplantation In Vivo

SERGEI A. KUZNETSOV,[1] PAUL H. KREBSBACH,[2,3] KAZUHITO SATOMURA,[1] JANET KERR,[1] MARA RIMINUCCI,[1] DAFNA BENAYAHU,[1] and PAMELA GEHRON ROBEY[1]

ABSTRACT

Populations of marrow stromal fibroblasts (MSFs) can differentiate into functional osteoblasts and form bone in vivo. It is not known, however, what proportion of MSF precursor cells, colony forming units-fibroblast (CFU-Fs), have osteogenic potential. In the present study, analysis of bone formation in vivo by single-colony derived strains of human marrow stromal fibroblasts (HMSFs) has been performed for the first time. Each strain originated from an individual CFU-F and underwent four passages in vitro prior to subcutaneous implantation into immunodeficient mice within vehicles containing hydroxyapatite-tricalcium phosphate ceramic. Multicolony derived HMSF strains were also transplanted to serve as positive controls. After 8 weeks, abundant bone formation was found in the transplants of all multicolony derived HMSF strains, whereas 20 out of 34 (58.8%) single-colony derived strains from four donors formed bone. Immunostaining with antibody directed against human osteonectin and in situ hybridization for human-specific *alu* sequences demonstrated that cells forming new bone were of human origin and were vital for at least 45 weeks post-transplantation. Both the incidence of bone-forming colonies and the extent of bone formation by single-colony derived HMSF strains were increased by cultivation with dexamethasone and ascorbic acid phosphate. Other factors, including type of transplantation vehicle, morphology, size, and structure of the original HMSF colonies showed no obvious correlation with the incidence or extent of bone formation. Hematopoietic tissue within the newly formed bone was developed in the transplants exhibiting exuberant bone formation. These results provide evidence that individual human CFU-Fs have osteogenic potential and yet differ from each other with respect to their osteogenic capacity. (J Bone Miner Res 1997;12:1335–1347)

INTRODUCTION

Marrow stromal fibroblasts (MSFs) are the in vitro progeny of a stromal cell type residing in the medullary cavity of bone.[1] In addition to their morphology, MSFs demonstrate a variety of other fibroblastic features and lack the basic characteristics of endothelial cells and macrophages.[2–5] Even after extended culture, MSFs can form bone, cartilage, fibrous tissue, adipose tissue, hematopoiesis-supporting reticular stroma,[1,6–10] and muscle.[11] Thus, the MSF population contains precursor cells capable of proliferation, renewal, and differentiation into several phenotypes.[1] One notable question that arises is whether these precursor cells are pluripotential and homogenous, that is, true stem cells, or whether they are a mixture of cells committed to various lineages of differentiation. In particular, it is not known if all or only some of the MSF precursors are osteogenic.

Clonal analysis provides a unique approach to characterizing the composition of a marrow stromal cell population. Immortalized clonal cell lines derived from bone and bone marrow stroma have been shown to vary widely in their

[1]Craniofacial and Skeletal Diseases Branch, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, U.S.A.
[2]Laboratory of Developmental Biology, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, U.S.A.
[3]Present address: Department of Oral Medicine/Pathology/Surgery, University of Michigan, School of Dentistry, Ann Arbor, Michigan, U.S.A.

Table 1. Bone Marrow Donors Used to Produce Multicolony Derived and Single-Colony Derived HMSF Strains

Donor	Gender, age (years)	Source of bone/bone marrow	Diagnosis	HMSF strains transplanted: Multicolony derived	HMSF strains transplanted: Single-colony derived
1	M, 46	aspirate, iliac crest	healthy volunteer	1	4
2	M, 13	surgical, iliac crest	subluxation of the 1st and 2nd cervical vertebrae	—	4
3	F, 5	surgical, ileum protrusion	cerebral palsy	2	15
4	M, 10	surgical iliac crest	spina bifida	2	11

ability to undergo osteogenic, chondrogenic, myogenic, and adipocytic differentiation, as well as to provide support for hematopoiesis and lymphopoiesis.[12–22] In contrast, primary colonies of mouse marrow stroma have been shown to uniformly support myelopoiesis and lymphopoiesis in vitro.[23] The observed phenotypic heterogeneity of long-term stromal cell lines is most likely linked to ongoing culture adaptation,[23] or to "capture" of different stages of developmental commitment,[15] and does not represent the natural structure of the stromal cell population. Moreover, the majority of continuous stromal cell lines, particularly those of human origin, grow in vitro without signs of senescence or degeneration[14,15,17,18,21,24–26] and do not represent their normal counterparts with respect to proliferation potential. To explore the natural organization of the marrow stromal cell population, it is necessary to study the original precursor cells, or their closest descendants, the nonimmortalized clones of MSFs.

In the present study, we have analyzed the osteogenic potential of nontransformed, nonimmortalized clonal human MSF strains. Each strain was produced from an individual, primary MSF colony. After four passages in vitro, each strain was transplanted subcutaneously into immunodeficient mouse recipients within vehicles containing hydroxyapatite-tricalcium phosphate (HA/TCP) ceramic. Bone formation was studied in the transplants harvested 8 weeks later. We demonstrate that individual, single-colony derived nontransformed strains of human MSFs exhibit osteogenic capacity. These strains differ widely with respect to their osteogenic potential, with more than a half of them forming bone after transplantation in vivo.

MATERIALS AND METHODS

In the present study, we chose to use an in vivo analysis of HMSF osteogenic capacity rather than to employ an in vitro approach based on biochemical markers of osteogenesis such as cAMP response to hormones. Although those criteria are important for cell characterization, an assay of bone formation in vivo is the most definitive determination of osteogenesis. HMSFs were grown in cultures of human marrow cell suspensions, passaged in vitro, loaded into transplantation vehicles, and transplanted subcutaneously into immunodeficient mouse recipients. The transplants were analyzed by means of histology, immunohistochemistry, and in situ hybridization.

Preparation of bone marrow single cell suspensions

Bone marrow specimens were obtained from four persons, in accordance with National Institutes of Health regulations governing the use of human subjects under protocol 94-D-0188. Donor information is summarized in Table 1. The bone marrow aspirate (0.5 cm^3) was collected with informed consent, using local anesthesia. The aspirate was mixed with 5 ml of ice-cold α-modified minimum essential medium (α-MEM, Life Technologies, Grand Island, NY, U.S.A.) containing 100 U/ml sodium heparin (Fisher Scientific, Fair Lawn, NJ, U.S.A.). The cells were centrifuged at 135*g* for 10 minutes, and the pellet was resuspended in fresh α-MEM. Surgical specimens containing fragments of normal unaffected bone with bone marrow were obtained from patients undergoing reconstructive surgery under IRB approved procedures. Fragments of trabecular bone and marrow were scraped gently with a steel blade into α-MEM and pipetted repeatedly to release the marrow cells. The marrow cell preparations from aspirate and surgical specimens were passed consecutively through 16 and 20 gauge needles to break up cell aggregates. The resulting cell suspensions were filtered through a 2350 nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) to remove any remaining cell aggregates.

Preparation of multicolony derived human marrow stromal fibroblast strains

Marrow single cell suspensions were plated at the following densities: 4.7×10^5 nucleated cells per square centimeter for aspirate, and $0.02–1.7 \times 10^5$ nucleated cells/cm^2 for surgical specimens. The plating cell density was higher for aspirate because the presence of peripheral blood cells lowered the colony forming efficiency. Cells were cultured in 75 cm^2 flasks or in 150-mm dishes (Becton Dickinson, Lincoln Park, NJ, U.S.A.) containing 30–50 ml of a growth medium consisting of α-MEM, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Biofluids, Rockville, MD, U.S.A.), and 20% fetal bovine serum (FBS; Life Technologies or Atlanta Biological, Norcross, GA, U.S.A.). Where indicated, growth medium was supplemented with 10^{-8} M dexamethasone (Dex; Sigma, St. Louis, MO, U.S.A.) and 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (AscP; Wako, Osaka, Japan). Cells were cultured at 37°C in an atmosphere of 100% humidity and 5% CO_2. Medium was replaced on day 1 for

aspirate cultures and on day 7 and 14 for all cultures, if not passaged by day 14.

In these primary cultures, HMSFs grew as discrete colonies (see below). To produce multicolony derived strains, we used cultures with high numbers of HMSF colonies (more than 100 per flask), and during the first passage, all the colonies were combined. First passage was performed on days 12–16 when colonies began to merge. The adherent cells were washed twice with Hank's balance salt solution (HBSS, Life Technologies) and treated with two consecutive portions of 1× trypsin-EDTA (Life Technologies) for 10–15 minutes each at room temperature. Cells were plated in 75 or 175 cm^2 flasks at $0.1–0.2 \times 10^5$ cells/cm^2. Subsequent passages were performed when cells were approaching confluence, usually 3–5 days later. For transplantation, multicolony derived HMSF strains of second or third passage were used. In total, 22 transplants of five multicolony derived HMSF strains established from three donors were performed.

Preparation of single-colony derived HMSF strains

For single-colony derived HMSF strains, marrow cells were plated into 150-mm dishes at densities of $0.14–14.0 \times 10^3$ nucleated cells/cm^2 for aspirate and $0.007–3.5 \times 10^3$ nucleated cells/cm^2 for surgical specimens. To ensure that plates contained well separated colonies, cells were plated at three concentrations, each differing by an order of magnitude. To prepare single-colony derived strains, cultures with between 10 and 30 HMSF colonies per dish were used. After 13–16 days, individual HMSF colonies separated by a distance of no less than two colony diameters were chosen. To exclude the possibility of combining two merging colonies, only colonies that featured an absolutely round shape were used. After two washes with HBSS, colonies were passaged using one of the following two techniques. First, a selected colony was removed from the plastic with a cell scraper. The tiny cell layer film was transferred to a Micro tube (PGC Scientifics, Frederick, MD, U.S.A.) and was treated with 1× trypsin-EDTA for 10–15 minutes at room temperature. In the second procedure, cloning cylinders were attached to the dish with the S/P High Vacuum Grease (Baxter Healthcare Corp., McGaw Park, IL, U.S.A.) so that each cylinder embraced an individual colony, and cells inside the cylinder were treated with two consecutive aliquots of 1× trypsin-EDTA for 5–10 minutes each at room temperature. In both cases, the released cells were transferred to individual wells of 6-well plates (Becton Dickinson). Subsequent passages were performed before cells reached confluence, usually 5–10 days later. Each strain was passaged consecutively to 25 cm^2 flask (second passage) and to 75 cm^2 flask (third passage). For transplantation, single-colony derived strains of the fourth passage were used. From two to four transplants per single-colony derived HMSF strain were performed. Altogether, 79 transplants of 34 single-colony derived HMSF strains established from four donors were successfully harvested.

Characterization of transplanted cell populations

To characterize the transplanted cells morphologically and cytochemically, a multicolony derived HMSF strain of the third passage and single-colony derived HMSF strains of the fourth passage were plated in two-well chamber slides (VWR Scientific, West Chester, PA, U.S.A.) at 0.5×10^5 cells/well. After 24 h, the cultures were stained for α-naphthyl acetate esterase and acid phosphatase activities (Sigma kits 91-A and 387-A, correspondingly) to determine the presence of nonfibroblastic cells. The number of cells positive and negative for each enzyme was determined by counting at least 200 cells in each of five separate regions of the slides. In parallel experiments, the multicolony and single-colony derived HMSF strains were analyzed by flow cytometry using forward scatter versus side scatter, as measures of cell size and granularity, with a FACScan (Becton Dickinson, Mansfield, MA, U.S.A.).

Preparation of human foreskin fibroblasts

Human foreskin fibroblasts (HFFs) were kindly provided by Dr. Mark DeNichilo. Up to the third passage, HFFs were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) containing 2 mM L-glutamine, 50 mg/ml streptomycin sulfate, 50 U/ml penicillin, and 10% FBS (HyClone, Logan, UT, U.S.A.). Cells of the third passage were transferred to the regular growth medium, and one more passage was performed. HFFs of the fourth passage were used as control cells for the transplantation studies. As other controls, vehicles without cells were also transplanted.

Loading cells into transplantation vehicles

We have found earlier that the most supportive vehicles for bone formation by multicolony derived HMSF strains are hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder and Collagraft, which is a mixture of HA/TCP powder and type I bovine fibrillar collagen.[27] These vehicles (Zimmer, Warsaw, IN, U.S.A.) have been shown to surpass HA/TCP blocks used by others[28–30] in terms of both efficiency of cell loading and extent of osteogenesis. We used $1.0–5.0 \times 10^6$ multicolony derived HMSFs, $1.5–3.0 \times 10^6$ single-colony derived HMSFs, or 3×10^6 HFFs per vehicle. For loading cells into both HA/TCP powder and Collagraft vehicles, novel methods have been developed, different from those used for HA/TCP blocks. Briefly, for a single transplant, 40 mg of HA/TCP powder, or a cubic block of Collagraft, 50–70 mm^3 volume, were used. Trypsin-released cells were pelletted in 1.7-ml Micro tubes at 135*g* for 10 minutes, and the cell pellet was resuspended in growth medium (in 1 ml for HA/TCP powder and in 30–60 μl for Collagraft). The powder was mixed with the cell suspension and incubated at 37°C for 70–100 minutes with slow rotation (25 rpm). The particles were collected following a brief centrifugation and supernatant was discarded. Collagraft was wetted with growth medium, dried briefly between sterile filter papers, and immediately placed into the cell suspension; as the vehicle expanded, it bound most of the cells. Prior to transplantation, the Collagraft vehicles with cells were incubated at 37°C for 60–90 min-

utes. It was estimated by cell counts that, after either procedure, greater than 80% of cells were incorporated into the vehicles.

Transplantation procedure

Immunodeficient 8- to 15-week-old female beige mice (NIH-bg-nu-xidBR, Charles River Laboratories, Raleigh, NC, U.S.A. or Harlan Sprague Dawley, Indianapolis, IN, U.S.A.) were used as subcutaneous transplant recipients. Operations were performed in accordance to specifications of an approved small animal protocol (114-93) under anesthesia achieved by intraperitoneal injection of 2.5% tribromoethanol (Sigma) at 0.018 ml/g of body weight. Midlongitudinal skin incisions of about 1 cm in length were made on the dorsal surface of each mouse, and subcutaneous pockets were formed by blunt dissection. A single transplant was placed into each pocket with up to four transplants per animal. The incisions were closed with surgical staples.

Fixation, histological examination, and evaluation of bone formation

The transplants were recovered 8 weeks post-transplantation, unless otherwise stated. Each transplant was cut into two to five pieces, fixed, and partially decalcified for 2 days in Bouin's solution (pH 1.6, Sigma). The transplants were then transferred to 70% ethanol and embedded into a single paraffin block so that the largest surface areas were sectioned. A section was prepared close to the block surface, then two consecutive steps of 0.5 mm each were made in the block, and sections were prepared at each of the steps. Sections were deparaffinized, hydrated, and stained with hematoxylin and eosin. Bone formation was blindly semiquantitatively estimated by three independent investigators (S.K., K.S., and M.R.). The following scale was applied to estimate bone formation in the transplants:

4+: abundant bone formation, bone spreads over more than one-half of the sections;
3+: moderate bone formation, bone structures occupy a significant part, but less than one-half of the sections;
2+: weak bone formation, bone structures occupy a small part of each section or of some sections;
1+: poor bone formation, a single, small bone trabecula found in only a few sections;
0: no signs of bone formation.

The average score of bone formation was calculated for each transplant and for each HMSF strain. The data were statistically analyzed using the Wilcoxon rank sums test which is based on the ordinal nature of the bone formation scores in the transplants. Differences were considered statistically significant at $p < 0.05$.

Immunostaining for human osteonectin

Immunohistochemical staining for human osteonectin was performed to identify the origin of the bone tissue formed in the transplants. Rabbit anti-human osteonectin antibody (HON), which does not cross-react with mouse osteonectin, was kindly provided by Dr. L.W. Fisher (CSDB, NIDR, NIH).[31] After deparaffinization and rehydration, the sections were incubated in 0.13% pepsin and 0.01 N HCl for 1 h at 37°C to reactivate the antigenicity of osteonectin. Indirect immunohistochemistry was carried out using HON (1:100) as primary antibody. Goat anti-rabbit IgG antibody (1:200, Kirkegaard and Perry Lab., Gaithersburg, MD, U.S.A.) was used as secondary antibody. Mouse vertebral bone and femoral bone of human fetus were used for negative and positive controls, respectively. As another negative control, normal rabbit serum (Vector Labs, Burlingame, CA, U.S.A.) replaced the primary antibody.

In situ *hybridization for human-specific* alu *sequence*

The human-specific repetitive *alu* sequence, which comprises about 5% of the total human genome, can be applied for identification of human cells.[32] We used in situ hybridization for *alu* sequence to study the origin of tissues formed in the transplants. The digoxigenin-labeled probe specific for *alu* sequence was prepared by PCR containing 1× PCR buffer (Perkin Elmer, Foster City, CA, U.S.A.), 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP, 0.065 mM dTTP, 0.035 mM digoxigenin-11-dUTP (Boehringer Mannheim Corp., Indianapolis, IN, U.S.A.), 10 pmol of specific primers, and 100 ng of human genomic DNA. The following primers were created on the basis of previously reported sequences[33]: sense, 5′-GTGGCTCACGCCTGTAATCC-3′, and antisense, 5′-TTTTTTGAGACGGAGTCTCGC-3′. The novel method of in situ hybridization was originally developed for use in decalcified sections of transplants in HA/TCP-containing vehicles. Sections deparaffinized with xylene and ethanol were immersed in 0.2 N HCl at room temperature for 7 minutes and then incubated in 1 mg/ml pepsin in 0.01 N HCl at 37°C for 10 minutes. After washing in PBS, the sections were treated with 0.25% acetic acid containing 0.1 M triethanolamine (pH 8.0) for 10 minutes and prehybridized with 50% deionized formamide containing 4× SSC at 37°C for 15 minutes. The sections were then hybridized with 1 ng/μl digoxigenin-labeled probe in hybridization buffer (1× Denhardt's solution, 5% dextrane sulfate, 0.2 mg/ml salmon sperm DNA, 4× SSC, 50% deionized formamide) at 42°C for 3 h after the denaturation step at 95°C for 3 minutes. After washing with 2× SSC and 0.1× SSC, digoxigenin-labeled DNA was detected by immunohistochemistry using antidigoxigenin alkaline phosphatase-conjugated Fab fragments (Boehringer Mannheim Corp.).

RESULTS

Development of MSF colonies in human marrow cell primary cultures

When single cell suspensions of human marrow were first plated at low cell densities, most adherent cells demonstrated macrophage-like morphology; among them, isolated cells of fibroblastic morphology could be observed. Some of the fibroblastic cells never proliferated, and together with

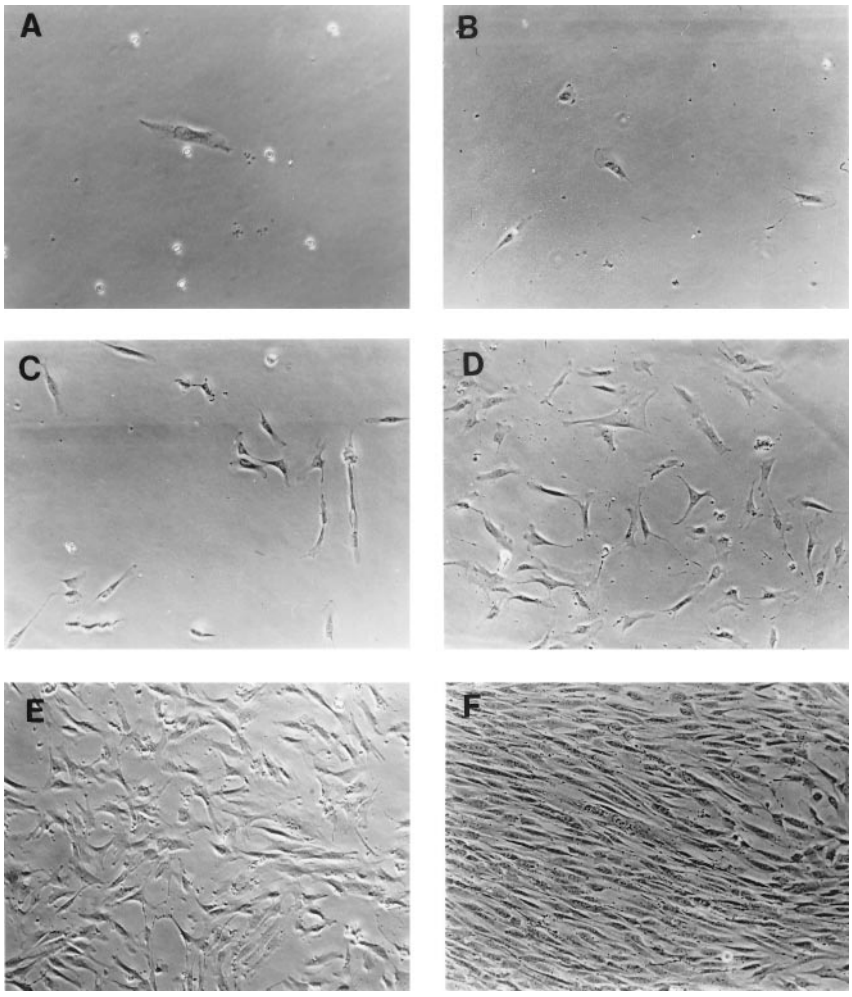

FIG. 1. Development of an individual HMSF colony in human bone marrow aspirate culture. The cells were plated at $0.14 \times 10^3/cm^2$, and the culture was observed by light microscopy. (A) A single fibroblast 1 day after explantation. (B) At 4 days, the colony consisted of four cells. (C) Part of the same colony at 6 days; the colony consisted of 45 cells. (D) Part of the same colony at 8 days; the colony consisted of about 150 cells. (E) Central part of the same colony at 10 days; the colony contained several hundred cells. (F) Peripheral part of the same colony at 14 days; the colony consisted of more than thousand cells. (A–F) Unstained cultures. (A) Magnification ×165; (B–F) ×82.5.

the majority of nonfibroblastic cells were lost from the culture. However, other fibroblastic cells underwent mitosis between days 1 and 3 and rapidly proliferated thereafter. From days 6 to 9, colonies could be observed consisting of several dozen to a few hundred loosely arranged fibroblasts. Later, the colonies acquired a dense, often multilayered structure, sometimes with spiral and nodule-like patterns. In 13- to 16-day cultures, small (up to 200 cells), average (from 200 to 1000 cells), and large (over 1000 cells) colonies were seen. Figure 1 shows the development of a HMSF colony from the initial single cell stage (Fig. 1A) through consecutive stages of cell proliferation (Figs. 1B–1E) to more than a thousand fibroblasts on day 14 (Fig. 1F). Based on the cell morphology, at least three types of HMSF colonies could be distinguished. The great majority of the colonies consisted of spindle-shaped HMSFs (spindle-shaped colonies) (Fig. 1F). The remaining colonies were composed of either very flattened HMSFs without bipolar orientation (flat colonies), or larger HMSFs whose shape was intermediate between spindle and flat (intermediate colonies).

Characteristics of HMSF strains

FACScan analysis using forward scatter to reflect cell size and side scatter to reflect cell granularity demonstrated that passaged HMSF strains contained three distinct cell fractions. The majority of the cells had a relative forward scatter of 300 to 600 and constituted a large cell fraction. The remaining cells had a relative forward scatter of 50 to 200 and constituted two small cell fractions different by cell granularity. Taken together, cells of the two small cell fractions constituted, in multicolony derived strain of the third passage, 1.4%, and in single-colony derived strain of the fourth passage, 1.3% of the total cell number.

The great majority of cells in HMSF strains had no detectable α-naphthyl acetate esterase or acid phosphatase activity. These cells exhibited a distinctive fibroblastic morphology: a large flattened cytoplasm and a large oval nucleus with prominent nucleoli (Fig. 2) and were MSFs.[3,4,34,35] Cells positive for α-naphthyl acetate esterase and cells positive for acid phosphatase constituted, correspondingly (mean ± SEM): in multicolony derived HMSF strain of the third passage, 1.2 ± 0.5 and 1.4 ± 0.4%;

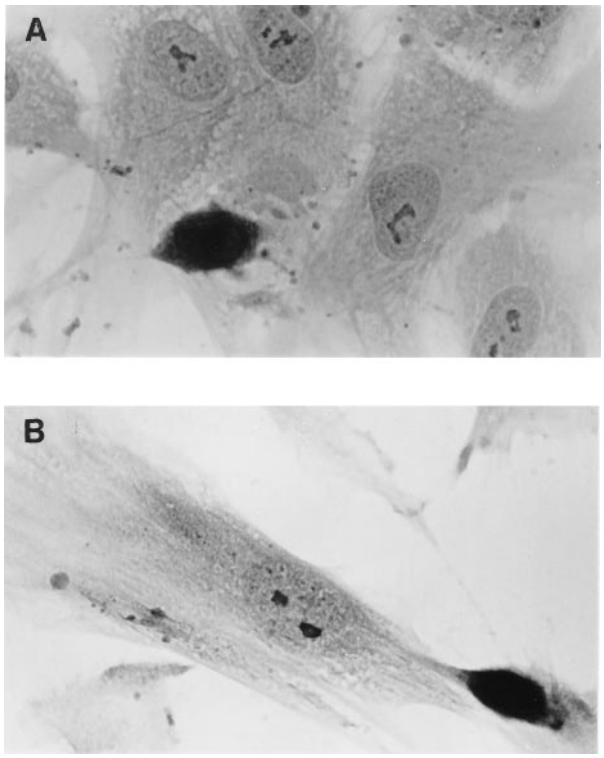

FIG. 2. Histochemical demonstration of enzyme activity in cells of HMSF strain. Large cells of fibroblastic morphology are negative for both α-naphthyl acetate esterase and acid phosphatase while small cells show high activity of both enzymes. (A) Fourth passage cells of single-colony derived HMSF strain stained for α-naphthyl acetate esterase. (B) Third passage cells of multicolony derived HMSF strain stained for acid phosphatase. (A, B) Counterstained with hematoxylin, magnification $\times$825.

in single-colony derived strain of the fourth passage, 1.2 $\pm$ 0.3% and 0.9 $\pm$ 0.2% of the total cell number. These cells were round, bipolar, or stellate in shape, were small, and had a dense nucleus (Fig. 2); they represented macrophages and endothelial cells.[4,35]

Morphology of the transplants

At 8 weeks post-transplantation, transplants of multicolony derived HMSF strains demonstrated exuberant bone formation (Fig. 3). In transplants of single-colony derived HMSF strains, a variable degree of bone formation could be observed (Fig. 4), ranging from abundant, interconnecting trabeculae in each section (score 4) to a tiny spicula in a few sections (score 1). In some transplants, only fibrous tissue was present with no evidence of bone formation (score 0). Bone formation originated on the surface of ceramic particles and progressed toward the interparticle spaces. The new bone contained embedded osteocytes and a prominent osteoblastic layer on lumenal surfaces. The spaces between bone trabeculae were filled with fibrous tissue (Figs. 3 and 4). In transplants featuring abundant bone formation, fields of hematopoiesis could be observed,

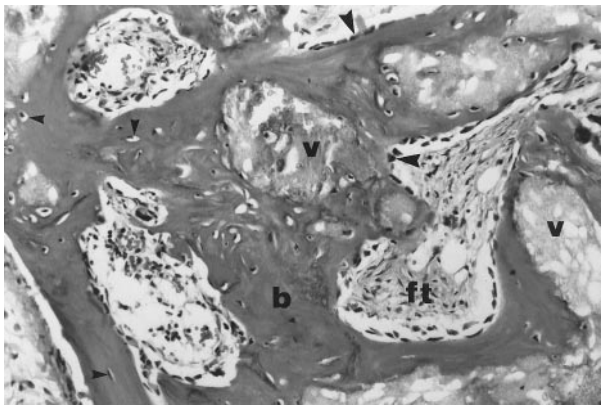

FIG. 3. Bone formation in transplant of multicolony derived HMSF strain harvested at 8 weeks. New bone (b) is formed on the surface of the vehicle particles (v); the spaces between bone trabeculae are filled with fibrous tissue (ft). The small arrowheads designate embedded osteocytes, the large arrowheads designate osteoblasts. Hematoxylin and eosin, magnification $\times$165.

always in close proximity to bone trabeculae. The hematopoietic tissue included reticular stroma, adipocytes, and numerous hematopoietic cells. Most of the hematopoietic cells were mature granulocytes of mouse origin, as judged by their morphology, though some immature cells and megakaryocytes could be also distinguished (Fig. 5). Hematopoietic tissue was observed in 100% of the transplants with a bone formation score of 3 or greater, in 80% of transplants with a score between 2 and 3, and in 33% of transplants with a score of 2. Hematopoiesis was never found in transplants with a bone formation score below 2. The abundance of the hematopoietic tissue also showed a positive correlation with the degree of bone formation.

Control transplants included vehicles loaded with human foreskin fibroblasts and vehicles without cells. In these transplants harvested 5–8 weeks post-transplantation, fibrous tissue growth was observed with no signs of osteogenesis or hematopoiesis (Fig. 6).

Bone formation in transplants of multicolony derived HMSF strains

New bone was formed in all transplants of multicolony derived HMSF strains (Table 2). In transplants originating from adult marrow aspirate (donor 1), the degree of bone formation increased from 5 to 45 weeks post-transplantation. In transplants originating from pediatric surgical specimens (donors 3 and 4), bone formation had achieved maximum scores at 8 weeks. The presence of Dex and AscP in growth medium had no influence on bone formation.

Bone formation in transplants of single-colony derived HMSF strains

Bone formation was found in transplants of 20 out of 34 single-colony derived HMSF strains (58.8%). Out of the 20 positive strains, 13 strains demonstrated bone formation in

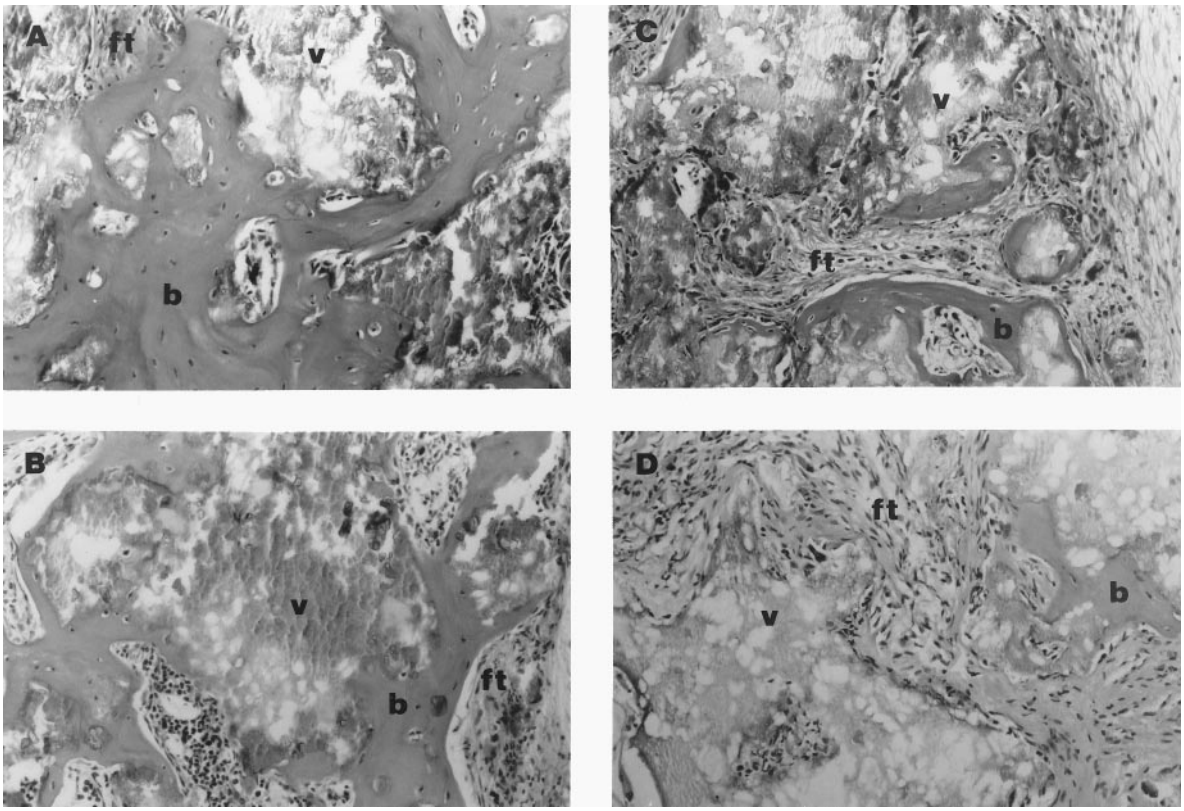

FIG. 4. Representative scores of bone formation in transplants of single-colony derived HMSF strains: (A) score 4, (B) score 3, (C) score 2, (D) score 1. The transplants harvested at 8 weeks demonstrate new bone (b), vehicle particles (v), and fibrous tissue (ft). (A–D) Hematoxylin and eosin, magnification ×165.

all transplants, while 7 other strains developed bone in some transplants but not in others. For 14 negative strains, none of the transplants showed any signs of osteogenesis.

Effect of transplantation vehicle: In transplants of single-colony derived HMSF strains, both HA/TCP powder and Collagraft proved to be equally supportive for bone formation (Table 3), although transplants in Collagraft generally contained high numbers of foreign body multinucleated cells.

Effect of Dex and AscP in growth medium: Single-colony derived HMSF strains were grown from day 0 either in the presence or in the absence of Dex and AscP in growth medium. For each of donors 2, 3, and 4, more single-colony derived strains formed bone, and the new bone was more extensive, when cultivation had been performed with Dex and AscP than without them (Table 4). Altogether, 68% of single-colony derived strains cultured with Dex and AscP proved to be osteogenic versus 33.3% of single-colony derived strains grown without the supplements. The difference between bone formation by strains cultured with and without Dex and AscP was statistically significant.

Dependence on bone marrow donor: For the four individual donors, positive strains constituted, consecutively, 2 out of 4 (50%), 2 out of 4 (50%), 8 out of 15 (53.3%), and 8 out of 11 (72.7%). Strains originated from donor 4 also formed more abundant bone, and more often formed bone accompanied by hematopoiesis, than strains originated from three other donors (Tables 3 and 4). The data presented here are not sufficient, however, to make any definitive conclusions about influence of donor age, gender, etc. on capability of HMSF strains to form bone and hematopoietic microenvironment.

Correlation of bone formation with HMSF morphology, colony size, and structure: We correlated bone-forming capacity of single-colony derived HMSF strains with morphological and proliferative characteristics of the original HMSF colonies. According to our morphological classification of the colonies, bone was formed by 15 out of 25 strains derived from spindle-shaped colonies (60%), by 2 out of 4 strains derived from flat colonies (50%), and by 3 out of 5 strains derived from intermediate colonies (60%). Likewise, the size and structure of the original colonies were not overtly correlated with the bone-forming potential (Table 5). In addition to colony size, proliferative capacity of the strains was reflected by their growth rate in passaged cultures. However, no direct correlation was found between this parameter and the occurrence of bone formation (not shown).

Among 20 single-colony derived HMSF strains positive for bone formation, extensive bone accompanied by hematopoietic tissue was formed by eight strains. Out of them, seven strains originated from spindle-shaped colonies and one strain from intermediate colony. Also, four out of the eight strains originated from large multilayered colonies

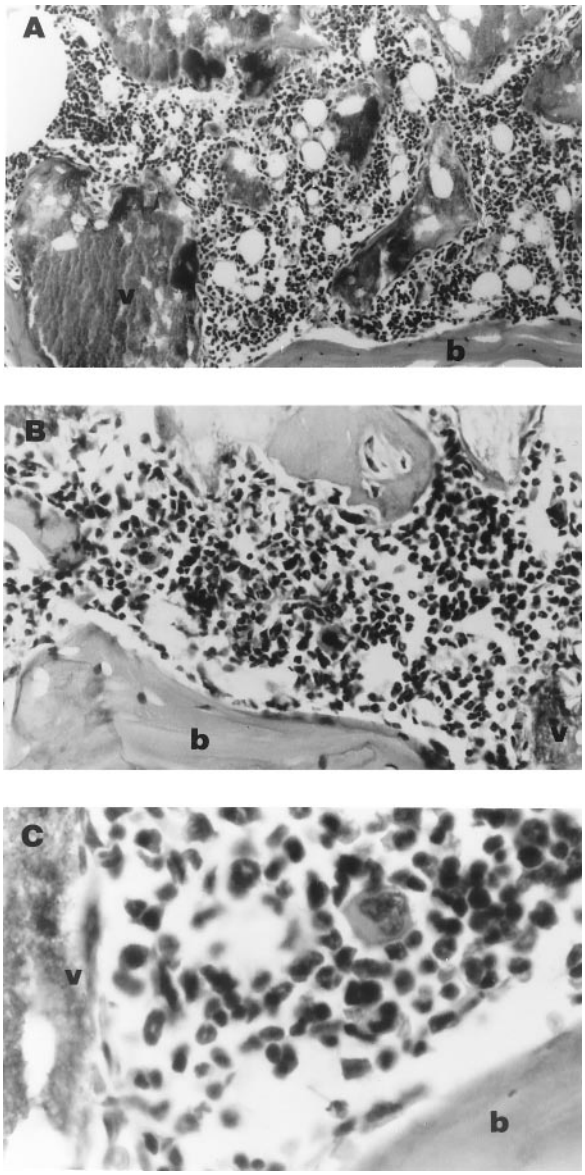

FIG. 5. Hematopoietic tissue in transplants of single-colony derived HMSF strains harvested at 8 weeks. Hematopoietic tissue is adjacent to the new bone (b); vehicle particles (v) are also seen. Note the presence of adipocytes in (A) and of megakaryocyte-like cells in (B) and (C). (A–C) Hematoxylin and eosin. (A) Magnification ×165; (B) ×330; (C) ×825.

with nodules, two strains from large multilayered colonies without nodules, and two strains from average monolayered colonies.

Confirmation of human origin of the new bone

To confirm that the cells of the new bone were of human origin, their responsiveness to antihuman osteonectin antibody was studied. In transplants of multicolony derived and single-colony derived HMSF strains recovered 8 weeks after transplantation, cells of the new bone demonstrated

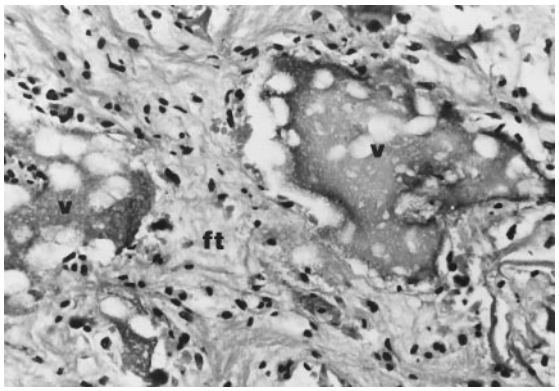

FIG. 6. Control transplant of human foreskin fibroblasts harvested at 7 weeks. Vehicle particles (v) are surrounded by fibrous tissue (ft) with no evidence of bone formation or hematopoiesis. Hematoxylin and eosin, magnification ×330.

intense immunostaining with anti-human osteonectin antibody (compare Figs. 7A and 7B with Fig. 7C). No immunoreactivity above background levels was seen in the tissues surrounding the transplants. To ascertain if the origin of the bone cells was changed with time, transplants of multicolony derived HMSF strain were harvested 45 weeks post-transplantation. At this late time stage, osteocytes were positive for human osteonectin (compare Figs. 7D and 7E). Immunoreactivity to human osteonectin was not observed in sections of mouse vertebral bone (Fig. 7F).

In transplants of multicolony derived HMSF strain harvested 45 weeks post-transplantation, the origin of tissues was studied by in situ hybridization for human-specific *alu* sequence. In these transplants, osteocytes and fibrous tissue cells were positive for *alu* sequence (Figs. 8A and 8B). *Alu* sequence was not detected in hematopoietic cells or in muscle cells that were located at the periphery of the transplants (not shown).

DISCUSSION

Origin of bone in transplants of HMSFs

When marrow-derived cells are transplanted into an open system, the question inevitably arises as to the origin, donor versus recipient, of a new bone. In the present study, two techniques were used to identify the origin of bone formed in transplants of HMSFs: immunocytochemical staining with antibody against human osteonectin, and in situ hybridization for human-specific *alu* sequence. Both methods show that the new bone in transplants of HMSFs is formed by implanted cells of human origin, rather than by cells of the mouse recipients. This observation held true for both multicolony derived and single-colony derived HMSF strains. Moreover, our results show that there was no replacement by bone cells of recipient origin in the transplants of HMSFs for at least 45 weeks after transplantation. Thus, in transplants of HMSFs, the new bone is self-maintained and is formed exclusively by transplanted donor

Table 2. Bone Formation in Transplants of Multicolony Derived HMSF Strains

Donor	Dex/AscP in growth medium	Passage number	Number of HMSFs per vehicle ($\times 10^6$)	Time after transplantation (weeks)	Bone formation score in the transplants		
					HA/TCP powder	Collagraft	Average
1	+	3	5.0	5	1.7	1.0	1.3
				8	2.0	1.3	1.6
				19	3.0	2.7	2.8
				45	3.5	3.2	3.3
3	+	2	2.0	8	3.7; 4.0	3.3; 4.0	3.7
	−		1.0	8	4.0	4.0	4.0
4	+	2	2.0	8	3.7; 4.0	2.3; 4.0	3.5
	−		2.0	8	3.7; 3.7	4.0; 4.0	3.8

Multicolony derived strains of human marrow stromal fibroblasts were transplanted within hydroxyapatite/tricalcium phosphate (HA/TCP) powder or Collagraft. The transplants were harvested 5–45 weeks post-transplantation. The following scale was used to estimate bone formation in the transplants:

4+: abundant bone formation, bone spreads over more than one-half of the sections;
3+: moderate bone formation, bone structures occupy a significant part, but less than one-half of the sections;
2+: weak bone formation, bone structures occupy a small part of each section or of some sections;
1+: poor bone formation, a single, small bone trabecula found in only a few sections;
0: no signs of bone formation.

Table 3. Bone Formation in Transplants of Single-Colony Derived HMSF Strains: Effect of Transplantation Vehicle

Donor	Number of transplants	Vehicle	Bone formation score				
			0	0–1	1.1–2	2.1–3	3.1–4
1	4	HA/TCP	4				
	6	Collagraft	4		1	1	
2	4	HA/TCP	3				1
	2	Collagraft			1	1	
3	13	HA/TCP	7	2	4		
	23	Collagraft	13	6	4		
4	12	HA/TCP	3		2	2	5
	15	Collagraft	4	1	2	3	5
Total	33	HA/TCP	17	2	6	2	6
	46	Collagraft	21	7	8	5	5

HMSF strains from four donors were transplanted within HA/TCP powder or Collagraft. The transplants were harvested 8 weeks later and the extent of bone formation was estimated. Difference in bone formation between transplants in HA/TCP powder and in Collagraft was not statistically significant ($p = 0.99$).

osteogenic cells. This conclusion is further supported by the absence of osteogenesis in transplants of human foreskin fibroblasts, or of empty vehicles. Our results also demonstrate that fibrous tissue formed in the HMSF transplants is of donor origin. In contrast, hematopoietic cells recruited to the transplants, as well as muscle tissue found at the periphery of the transplants, are of recipient origin.

Osteogenic and nonosteogenic colony forming units-fibroblast

We supported, by direct observations of HMSF colony development, the earlier data that each MSF colony is a cell clone originated from a single precursor cell termed the colony forming unit-fibroblast (CFU-F).[2,36,37] To ensure that single-colony derived HMSF strains were in fact derived from individual colonies, extreme precautions were taken to prevent HMSF colony cross-contamination. These measures included very low plating cell densities, careful selection of colonies by shape and location and avoidance of mechanical disturbances to the cultures. However, it should be recognized that there are no markers available to verify directly the clonal origin of the single-colony derived HMSF strains used in this study. In other studies where there was a known genetic somatic mutation expressed in one of two alleles, we have verified by direct DNA sequencing the

Table 4. Bone Formation in Transplants of Single-Colony Derived HMSF Strains: Effect of Dexamethasone and Ascorbic Acid Phosphate Salt in the Growth Medium

Donor	Number of strains	Dex/AscP in medium	Bone formation score 0	0–1	1–2	2–3	3–4
1	4	+	2	1		1	
2	3	+	1	1		1	
	1	−	1				
3	10	+	4	3	3		
	5	−	3	2			
4	8	+	1		1	2	4
	3	−	2		1		
Total	25	+	8	5	4	4	4
	9	−	6	2	1		

Cells of single-colony derived HMSF strains were cultured in growth medium with or without 10^{-8} M dexamethasone (Dex) and 10^{-4} M L-ascorbic acid phosphate magnesium salt n-hydrate (AscP). Following transplantation and harvesting (8 weeks), the extent of bone formation was estimated. Difference between bone formation by strains cultured with and without Dex/AscP was statistically significant ($p = 0.034$).

Table 5. Bone Formation in Transplants of Single-Colony Derived HMSF Strains: Effect of Size and Structure of Original HMSF Colonies

Colony size	Colony structure: Multilayered with nodules	Multilayered without nodules	Monolayered	Total
Large	8/12	4/8	2/2	14/22
Average	—	1/2	3/6	4/8
Small	—	—	2/4	2/4
Total	8/12	5/10	7/12	20/34

Values are number of strains positive for bone formation divided by the total number of strains. Colony size was defined at the time of the first passage as follows: small, up to 200 cells; average, between 200 and 1000 cells; large, over 1000 cells. The transplants of single-colony derived strains were harvested 8 weeks post-transplantation and evaluated for osteogenesis.

clonality of single-colony derived HMSF strains isolated using the identical technique (Kuznetsov, Riminucci, Gehron Robey, unpublished observation). We consider it highly probable that single-colony derived strains used in this study are of clonal origin, consequently their differentiation patterns may be used to draw conclusions about differentiation potential of their original CFU-Fs. The presence of a few nonfibroblastic cells in single-colony derived HMSF strains does not interfere with HMSF clonality because macrophages may be attracted and stimulated by HMSF colonies.

A unique feature of our investigation was that the closest possible descendants of the original CFU-Fs were used for transplantation. In this respect, our HMSF strains differ significantly from continuous stromal cell lines employed in most earlier studies.[12–22,24–26] Thus, our data more likely represent the characteristics of the original CFU-Fs. Using this approach we found for the first time that individual human CFU-Fs have osteogenic potential. We further demonstrated that clonal HMSF strains and original human CFU-Fs are heterogeneous with respect to their osteogenic capacity. Similar conclusions have been made earlier for MSFs of nonhuman origin. When mouse and guinea pig MSF colonies were transplanted autologously, about 20% of the colonies formed bone.[38] When rabbit single-colony derived MSF strains were transplanted intraperitoneally within diffusion chambers, either autologously or into immunodeficient mice, bone was formed in 48.3[37,39] and in 36.8%[10] of the transplants, respectively. In the present study, we demonstrated that greater than 50% of human single-colony derived MSF strains have osteogenic potential.

Our results show that single-colony derived HMSF strains vary in their differentiation capacities. After in vivo transplantation, some strains develop extensive bone and hematopoietic tissue, other strains form little bone without hematopoiesis, while the remaining strains form only fibrous tissue. These findings are similar to those observed in transplants of mouse and guinea pig primary MSF colonies.[38] It seems likely that those single-colony derived strains, or MSF colonies, which form both the bone and hematopoietic microenvironment, originate from pluripotent CFU-Fs, that is, from true stem cells. Strains that form only bone and/or fibrous tissue may originate from CFU-Fs which are already committed to a particular line of differentiation. If this assumption is valid, our findings lend considerable strength to the hypothesis that the marrow CFU-F population is divided into two compartments: stem cells and committed progenitors.[1]

Factors related to osteogenic capacity of transplanted HMSFs

In the present study, we compared the effects of several parameters on bone formation by transplanted HMSFs. These factors included different types of transplantation vehicles, presence or absence of Dex and AscP in growth medium, and morphological and proliferative characteristics of HMSFs. The two vehicles tested, HA/TCP ceramic powder and Collagraft, equally supported bone formation by single-colony derived HMSF strains.

The presence of Dex and AscP in growth medium had no effect on bone formation by multicolony derived HMSF strains, but significantly increased the incidence and extent of bone formation by single-colony derived HMSF strains. These results lend further support to our earlier data,[27] as well as to findings by others.[30,40,41] It has been shown that multicolony derived HMSF strains form bone regardless of cultivation with or without Dex and AscP if transplanted in HA/TCP-containing vehicles, but only after cultivation with Dex and AscP when transplanted in diffusion chambers or Gelfoam. Presumably, stimulation by Dex and AscP is not necessary for bone formation by strongly osteogenic multi-

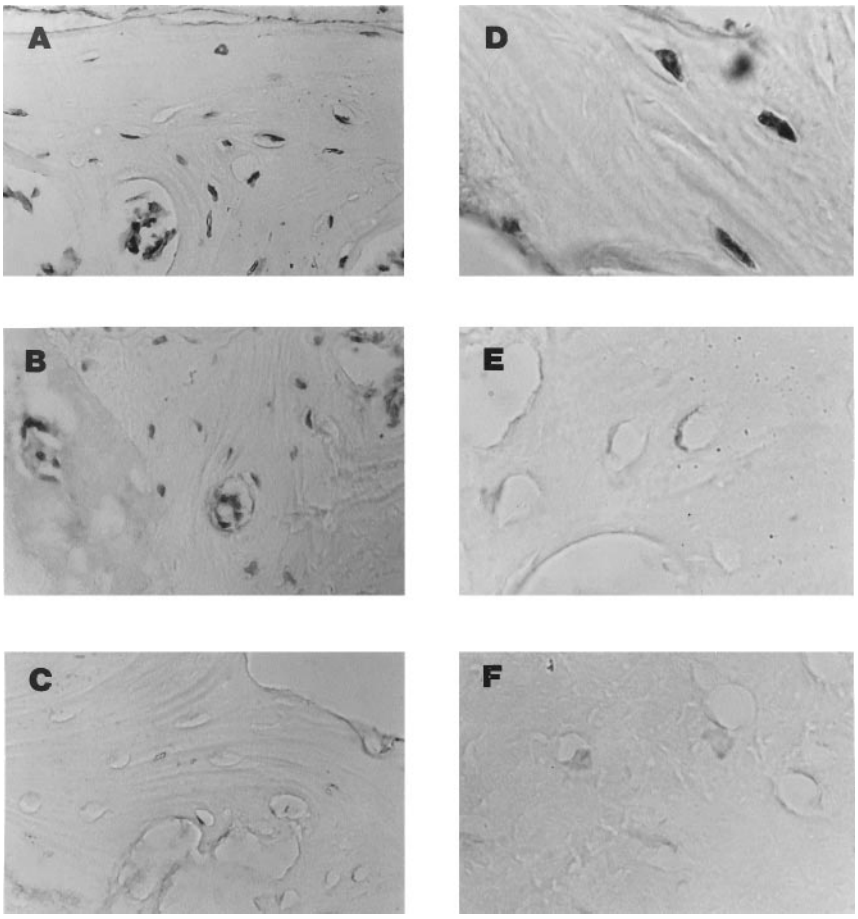

FIG. 7. Presence of human osteonectin in bone cells of HMSF transplants. (A) Multicolony derived strain harvested 8 weeks post-transplantation; immunostaining with anti-human osteonectin antibody (HON). (B) Single-colony derived strain harvested 8 weeks post-transplantation; immunostaining with HON. (C) Multicolony derived strain harvested 8 weeks post-transplantation; immunostaining with normal rabbit serum. (D) Multicolony derived strain harvested 45 weeks post-transplantation; immunostaining with HON. (E) Multicolony derived strain harvested 45 weeks post-transplantation; immunostaining with normal rabbit serum. (F) Section of mouse vertebral bone; immunostaining with HON. Note the presence of positively stained osteocytes in (A), (B), and (D), and absence of staining in (C), (E), and (F). (A–C) Magnification ×330; (D–F) ×825.

colony derived strains transplanted in optimal vehicles. However, if either cells are less osteogenic, such as some single-colony derived HMSF strains, or vehicles are less supportive of osteogenesis, Dex and AscP become necessary for bone formation. This conclusion complements earlier observations that Dex stimulates osteogenic differentiation of MSFs in vitro.[42–44] More generally, our data support the view that osteogenic differentiation of CFU-F descendants can be modified by exogenous factors.[45]

In an attempt to find distinctive features of osteogenic colonies, the original HMSF colonies were classified according to their size, structure, and cell morphology. However, none of these parameters nor the growth rate of resulting single-colony derived HMSF strains proved to be significant in predicting bone-forming capacity of the strains. It is concluded that if any of these parameters reflect intrinsic differences between the original CFU-Fs, these differences are not directly related to their osteogenic potential.

Relation between osteogenesis and support of hematopoiesis

Abundant hematopoietic tissue adjacent to the new bone was formed in the transplants of 8 out of 34 (23.5%) single-colony derived strains. At a morphological level, no definitive markers were found to distinguish those HMSF colonies that were capable to differentiate towards both osteogenesis and hematopoiesis support. The hematopoietic cells were of mouse origin and consisted mainly of mature granulocytes but included some immature cells and megakaryocytes. It seems, therefore, that in the transplants of HMSFs, mouse hematopoiesis was established in a human microenvironment. In vitro, a reverse relationship between differentiation of marrow stroma toward osteogenesis and hematopoiesis support has been described.[46] In HMSF transplants, on the contrary, the development of hematopoietic tissue always positively correlated with abundance of bone.

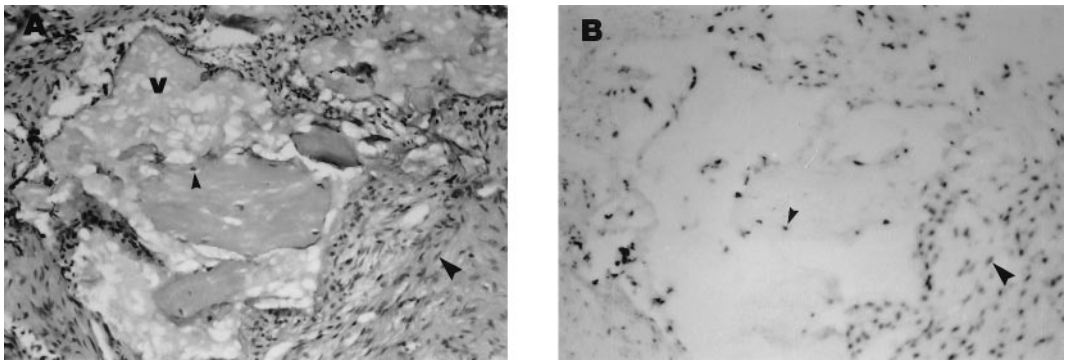

FIG. 8. Expression of human-specific *alu* sequence in transplant of multicolony derived HMSF strain harvested 45 weeks post-transplantation. (A) Hematoxylin and eosin. (B) The same field, in situ hybridization for *alu* sequence. Note that both osteocytes (small arrowheads) and fibrous tissue cells (large arrowheads) stain positively for human-specific *alu* sequence. V, vehicle. (A, B) Magnification ×165.

In conclusion, we have demonstrated that nontransformed, nonimmortalized, clonal strains of human marrow stromal fibroblasts can form bone after transplantation in vivo, and that the population of human marrow CFU-Fs is heterogeneous with respect to their osteogenic capacity. It is important, at the fundamental as well as the clinical level, to be able to distinguish osteogenic from nonosteogenic CFU-Fs in marrow cell suspension and osteogenic from nonosteogenic HMSF colonies in primary culture. Experiments in progress are aimed at identifying molecular markers that would facilitate isolation of osteogenic subsets of marrow stromal fibroblasts.

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REFERENCES

1. Owen M. 1988 Marrow stromal stem cells. J Cell Sci Suppl **10:**63–76.
2. Castro-Malaspina H, Gay RE, Resnick G, Kapoor N, Meyers P, Chiarieri D, McKenzie S, Broxmeyer HE, Moore MAS 1980 Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. Blood **56:**289–301.
3. Song ZX, Quesenberry PJ 1984 Radioresistant murine marrow stromal cells: A morphologic and functional characterization. Exp Hematol **12:**523–533.
4. Fei R-G, Penn PE, Wolf NS 1990 A method to establish pure fibroblast and endothelial cell colony cultures from murine bone marrow. Exp Hematol **18:**953–957.
5. Zhang RW, Supowit SC, Xu X, Li H, Christensen MD, Lozano R, Simmons DJ 1995 Expression of selected osteogenic markers in the fibroblast-like cells of rat marrow stroma. Calcif Tissue Int **56:**283–291.
6. Ashton BA, Allen TD, Howlett CR, Eaglesom CC, Hattori A, Owen M 1980 Formation of bone and cartilage by marrow stromal cells in diffusion chambers in vivo. Clin Orthop **151:**294–307.
7. Patt HM, Maloney MA, Flannery ML 1982 Hematopoietic microenvironment transfer by stromal fibroblasts derived from bone marrow varying in cellularity. Exp Hematol **10:**738–742.
8. Kuznetsov SA, Grosheva AG, Friedenstein AJ 1989 Osteogenic properties of adhesive cells from mouse bone marrow dexter cultures. Bull Exper Biol Med **108:**1186–1188.
9. Friedenstein AJ 1990 Osteogenic stem cells in the bone marrow. Bone Miner Res **7:**243–272.
10. Bennett JH, Joyner CJ, Triffitt JT, Owen ME 1991 Adipocytic cells cultured from marrow have osteogenic potential. J Cell Sci **99:**131–139.
11. Wakitani S, Saito T, Caplan AI 1995 Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. Muscle Nerve **18:**1417–1426.
12. Aubin JE, Heersche JNM, Merrilees MJ, Sodek J 1982 Isolation of bone cell clones with differences in growth, hormone responses, and extracellular matrix production. J Cell Biol **92:**452–461.
13. Kodama HA, Sudo H, Koyama H, Kasai S, Yamamoto S 1984 In vitro hemopoiesis within a microenvironment created by MC3T3-G2/PA6 preadipocytes. J Cell Physiol **118:**233–240.
14. Grigoriadis AE, Heersche JHM, Aubin JE 1990 Continuously growing bipotential and monopotential myogenic, adipogenic, and chondrogenic subclones isolated from the multipotential RCJ 3.1 clonal cell line. Dev Biol **142:**313–318.
15. Yamaguchi A, Kahn AJ 1991 Clonal osteogenic cell lines express myogenic and adipocytic developmental potential. Calcif Tissue Int **49:**221–225.
16. Hunt P, Robertson D, Weiss D, Rennick D, Lee F, Witte ON 1987 A single bone marrow-derived stromal cell type supports the in vitro growth of early lymphoid and myeloid cells. Cell **48:**997–1007.
17. Collins LS, Dorshkind K 1987 A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. J Immunol **138:**1082–1087.

18. Song Z, Thomas C, Innes D, Waheed A, Shadduck RK, Quesenberry PJ 1988 Characterization of two clones isolated from the TC-1 murine marrow stromal cell line: Growth factor and retrovirus production and physical support of hemopoiesis. Intern J Cell Cloning **6:**125–145.
19. Gimble JM, Pietrangeli C, Henley A, Dorheim MA, Silver J, Namen A, Takeichi M, Goridis C, Kincade PW 1989 Characterization of murine bone marrow and spleen-derived stromal cells: Analysis of leukocyte marker and growth factor mRNA transcript levels. Blood **74:**303–311.
20. Dorheim M-A, Sullivan M, Dandapani V, Wu X, Hudson J, Segarini PR, Rosen DM, Aulthose AL, Gimble JM 1993 Osteoblastic gene expression during adipogenesis in hematopoietic supporting murine bone marrow stromal cells. J Cell Physiol **154:**317–328.
21. Fried A, Benayahu D, Wientroub S 1993 Marrow stroma-derived osteogenic clonal cell lines: putative stages in osteoblast differentiation. J Cell Physiol **155:**472–478.
22. Benayahu D, Gurevitch O, Zipori D, Wientroub S 1994 Bone formation by marrow osteogenic cells (MBA-15) is not accompanied by osteoclastogenesis and generation of hematopoietic supportive microenvironment. J Bone Miner Res **9:**1107–1114.
23. Deryugina EI, Ratnikov BI, Bourdon MA, Muller-Sieburg CE 1994 Clonal analysis of primary marrow stroma: Functional homogeneity in support of lymphoid and myeloid cell lines and identification of positive and negative regulators. Exp Hematol **22:**910–918.
24. Singer JW, Charbord P, Keating A, Nemunatis J, Raugi G, Wight TN, Lopez JA, Roth GJ, Dow LW, Fialkow PJ 1987 Simian virus 40–transformed adherent cells from human long-term marrow cultures: Cloned cell lines produce cells with stromal and hematopoietic characteristics. Blood **70:**464–474.
25. Harigaya K, Handa H 1985 Generation of functional clonal cell lines from human bone marrow stroma. Proc Natl Acad Sci USA **82:**3477–3480.
26. Takahashi S, Reddy SV, Dallas M, Delvin R, Chou JY, Roodman GD 1995 Development and characterization of a human marrow stromal cell line that enhances osteoclast-like cell formation. Endocrinology **136:**1441–1449.
27. Krebsbach PH, Kuznetsov SA, Satomura K, Emmons RVB, Rowe DW, Gehron Robey P 1997 Bone formation in vivo: Comparison of osteogenesis by transplanted mouse and human marrow stromal fibroblasts. Transplantation **63:**1059–1069.
28. Dennis JE, Haynesworth SE, Young RG, Caplan AI 1992 Osteogenesis in marrow-derived mesenchymal cell porous ceramic composites transplanted subcutaneously: Effect of fibronectin and laminin on cell retention and rate of osteogenic expression. Cell Transplant **1:**23–32.
29. Goshima J, Goldberg VM, Caplan AI 1991 The origin of bone formed in composite grafts of porous calcium phosphate ceramic loaded with marrow cells. Clin Orthop **269:**274–283.
30. Haynesworth SE, Goshima J, Goldberg VM, Caplan AI 1992 Characterization of cells with osteogenic potential from human marrow. Bone **13:**81–88.
31. Fisher LW, Hawkins GR, Tuross N, Termine JD 1987 Purification and partial characterization of small proteoglycans I and II, bone sialoproteins I and II, and osteonectin from the mineral compartment of developing human bone. J Biol Chem **262:**9702–9709.
32. Jacobsen PF, Daly J 1994 A method of distinguishing human and mouse cells in solid tumors using in situ hybridization. Exp Mol Pathol **61:**212–220.
33. Matera AG, Hellmann U, Hintz MF, Schmid CW 1990 Recently transposed Alu repeats result from multiple source genes. Nucl Acid Res **18**(20)**:**6019–6023.
34. Wang QR, Wolf NS 1990 Dissecting the hematopoietic microenvironment. VIII. Clonal isolation and identification of cell types in murine CFU-F colonies by limiting dilutions. Exp Hematol **18:**355–359.
35. Penn PE, Jiang D-Z, Fei R-G, Sitnicka E, Wolf NS 1993 Dissecting the hematopoietic microenvironment. IX. Further characterization of murine bone marrow stromal cells. Blood **81:**1205–1213.
36. Latsinik NV, Gorskaya UF, Grosheva AG, Domogatskii SP, Kuznetsov SA, Narovlyanskii NN, Pavlenko RG, Trubetskaya OL, Friedenstein AJ 1986 Content of stromal colony-forming cells (CFUf) in mouse bone marrow and the clonal nature of fibroblast colonies formed by them. Sovjet J Dev Biol **17:**22–29.
37. Friedenstein AJ, Chailakhyan RK, Gerasimov YV 1987 Bone marrow osteogenic stem cells: In vitro cultivation and transplantation in diffusion chambers. Cell Tissue Kinet **20:**263–272.
38. Chailakhyan RK, Gerasimov YV, Friedenstein AJ 1978 Transfer of bone marrow microenvironment by clones of stromal mechanocytes. Bull Exper Biol Med **86:**1633–1635.
39. Gerasimov YV, Friedenstein AJ, Chailakhyan RK, Shishkova VV 1986 Powers of differentiation of clonal strains of bone marrow fibroblasts. Bull Exper Biol Med **101:**802–805.
40. Ashton BA, Abdullah F, Cave J, Williamson M, Sykes BC, Couch M, Poser JW 1985 Characterization of cells with high alkaline phosphatase activity derived from human bone marrow: Preliminary assessment of their osteogenicity. Bone **6:**313–319.
41. Gundle R, Joyner CJ, Triffitt JT 1995 Human bone tissue formation in diffusion chamber culture in vivo by bone-derived cells and marrow stromal fibroblastic cells. Bone **16:**597–601.
42. Leboy PS, Beresford JN, Devlin C, Owen ME 1991 Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. J Cell Physiol **146:**370–378.
43. Cheng S-L, Yang JW, Rifas L, Zhang S-F, Avioli LV 1994 Differentiation of human bone marrow osteogenic stromal cells in vitro: Induction of the osteoblast phenotype by dexamethasone. Endocrinology **134:**277–286.
44. Hebertson A, Aubin JE 1995 Dexamethasone alters the subpopulation make-up of rat bone marrow stromal cell cultures. J Bone Miner **10:**285–294.
45. Owen ME, Cave J, Joyner CJ 1987 Clonal analysis in vitro of osteogenic differentiation of marrow CFU-F. J Cell Sci **87:**731–738.
46. Van Vlasselaer P, Falla N, Van Den Heuvel R, Dasch J, de Waal Malefijt R 1995 Interleukin-10 stimulates hematopoiesis in murine osteogenic stroma. Clin Orthop N **313:**103–114.

Address reprint requests to:
Dr. Sergei A. Kuznetsov
National Institute of Dental Research
National Institutes of Health
Building 30, Room 106
30 Convent Dr MSC 4320
Bethesda, MD 20892-4320 U.S.A.

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